POPULATION STRUCTURE OF LOGGERHEAD SEA TURTLES (*Caretta caretta*) NESTING IN
THE SOUTHEASTERN UNITED STATES INFERRED FROM MITOCHONDRIAL DNA
SEQUENCES AND MICROSATELLITE LOCI

by

BRIAN MICHAEL SHAMBLIN

(Under the direction of Campbell J. Nairn and John P. Carroll)

ABSTRACT

The loggerhead sea turtle *Caretta caretta* is a globally threatened species. The south-
eastern United States of America (USA) nesting assemblage is one of the two primary log-
gerhead rookeries in the world and the largest in the Atlantic. Determining the stock structure
within this nesting aggregation is important for defining management units and providing
baseline data for mixed stock analyses of foraging aggregations and stranded turtles. I con-
ducted molecular analyses utilizing mitochondrial DNA (mtDNA) sequence and newly devel-
oped microsatellite primers on samples collected from nine sample sites from North Carolina
to the Florida panhandle to characterize the population structure of rookeries comprising the
southeastern USA nesting assemblage. Pairwise comparisons of mtDNA haplotype frequen-
cies among eight sample sites utilizing genetic distance-based and frequency-based models
suggested significant structure among most sites tested in Florida. Pairwise F-statistics and
R-statistics revealed no significant nuclear allele frequency differences among any of the nine
sample sites. This pattern of complex population structure may be attributable to migration-
mediated gene flow among nesting beaches that are discrete by virtue of female nest site
fidelity. However, pairwise tests of genotypic frequency divergence did suggest shallow but

significant differences between Georgia and Florida sites and South Carolina and Florida sites, consistent with the segregation of northern subpopulation and Florida loggerheads on foraging grounds supported by carapace epibiont and tagging studies. Analysis of molecular variance (AMOVA) and pairwise comparisons suggested that the currently designated south Florida management unit may be comprised of multiple management units. More extensive geographical sampling is required to make more robust inferences about the integrity and boundaries of management units.

INDEX WORDS: loggerhead sea turtle, *Caretta caretta*, population structure, mitochondrial DNA, microsatellites

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CHAPTER 1

INTRODUCTION

The loggerhead sea turtle (*Caretta caretta*) is one of seven extant species of marine turtles. It belongs to the superfamily Chelonioidea, which is comprised of the two extant marine turtle families, Cheloniidae and the monotypic Dermochelyidae. Loggerheads share the family Cheloniidae with the other "hard-shelled" marine turtle species: the green turtle (*Chelonia mydas*), hawksbill (*Eretmochelys imbricata*), Kemp's Ridley (*Lepidochelys kempii*), olive Ridley (*Lepidochelys olivacea*), and the flatback (*Natator depressus*).

Loggerheads nesting on western North Atlantic beaches exhibit a complex life history characterized by ontogenetic habitat shifts and seasonal migrations. After emerging from the nest, hatchlings undergo a swimming frenzy lasting for approximately 24 hours, which is thought to allow the hatchlings to reach the Gulf Stream (Wyneken and Salmon 1992). The duration of the oceanic juvenile stage for western North Atlantic loggerheads has been estimated at 6.5 to 11.5 years based on size-frequency distributions of tagged turtles from around the Azores (Bjorndal et al. 2000). During this stage, juveniles are largely epipelagic and may actively swim or passively drift with ocean currents (Bolten 2003). These turtles ultimately recruit to the neritic zone and are seasonally common in a variety of habitats along the continental shelf from New York to the Gulf of Mexico (Butler et al. 1987, Fritts et al. 1983, Morreale et al. 1992). Neritic juveniles and foraging adults migrate seasonally along the continental shelf in the Atlantic to thermoregulate, retreating south in the fall to escape cold water and moving northward in the spring as the water warms (Shoop and Kenney 1992). Similarly, loggerheads use water temperatures as a cue to move out of shallow estuarine systems and into open water (Ranaud and Carpenter 1994). Breeding adults also

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migrate between foraging grounds and breeding grounds associated with nesting beaches. Sex ratios of adult loggerheads captured in the Canaveral channel suggest strong seasonality of breeding migrations with the sex ratio of captures shifting from 24 males:1 female during the months of March to May to 1 male:32 females from June through August (Dickerson et al. 1995).

Loggerheads typically nest on more temperate beaches than the other marine turtle species (Bolten 2003). Nesting occurs in the Atlantic, Pacific, and Indian Ocean basins. The two primary nesting aggregations are the southeastern USA and the island of Masirah off Oman in the Indian Ocean, both of which host greater than 10,000 nesting females annually (TEWG 1998, Ross 1998). Within the western Atlantic, the southeastern USA nesting aggregate comprised of beaches from Virginia to Texas is the largest, with Florida accounting for roughly 90% of the nesting effort within the USA (Murphy and Hopkins 1984, Meylan et al. 1995). Moderate nesting also occurs in Yucatan and Quintana Roo in Mexico, as well as Brazil. In the eastern Atlantic, nesting occurs primarily in the eastern Mediterranean and on the Cape Verde islands off the western coast of Africa. The recently discovered Cape Verde population has proven at least an intermediate-sized rookery with over 1,000 females tagged on just 5 km of beach on Boa Vista alone during the 2000 nesting season (Ehrhart et al. 2003).

Fecundity data have been generated by numerous flipper-tagging, mark-recapture studies conducted on nesting beaches in the USA (Ehrhart 1980, Addison 1996, Williams and Frick 2001). These data have been used to estimate clutch frequency and remigration interval. Upon reaching sexual maturity, female loggerheads may lay between one and seven nests within a single nesting season (Dodd 1988). The number of years between nesting visits is known as the remigration interval. The remigration intervals for western North Atlantic log- gerheads range from one to 15 years, though annual nesting cycles are quite rare in the USA (Dodd 1988). The mean observed remigration interval at various sites on US nesting beaches ranges from approximately 2.5 to 3.0 years (Richardson et al. 1978, Bjorndal et al. 1983).

Parameter estimates for clutch frequency and remigration interval are strongly influenced by the site fidelity of nesting females and the ability to intercept and identify all females on the study beach (Schroeder et al. 2003), so these estimates should be treated with caution.

Early mark-recapture data from green turtle studies indicated that some of the females were returning to the same beach to nest in subsequent remigrations (Carr and Ogren 1960). This observation prompted a number of hypotheses to explain the ability of females to navigate from often distant foraging grounds to the same nesting beaches in different nesting seasons. Two hypotheses gained prominence: social facilitation and natal homing. The social facilitation hypothesis proposes that neophyte females follow experienced nesters from foraging grounds to nesting sites, and after successful nesting, imprint on that site for future nesting attempts (Hendrickson 1958, Owens et al. 1982). Carr (1967) suggested that females were returning to their natal rookeries to nest. The natal homing hypothesis proposes that hatchlings imprint on some aspect of the natal beach as they emerge from the nest and flee to the ocean. Direct tests of these hypotheses have been prohibitive because of the lack of a persistent tag able to withstand years of growth and abuse as hatchlings mature into reproductive adults (Carr 1986). Additionally, because of the apparently low recruitment rate, prodigious numbers of hatchlings would have to be marked.

Mitochondrial DNA (mtDNA) sequence variation has proven useful in examining intraspecific phylogeography (Avise et al. 1987), and provided an indirect test of the two hypotheses. The properties of non-assorting and direct maternal transmission make mitochondrial DNA polymorphisms particularly useful when matrilines are of interest, which is the case when examining population structure among marine turtle rookeries. Investigations of green turtle global phylogeography revealed fixed haplotype differences and significant structuring of haplotype frequencies among several rookeries, providing strong evidence for natal homing (Meylan et al. 1990, Bowen et al. 1992). Analysis of loggerhead mtDNA restriction-sites revealed shallow but significant differences among rookeries in the

Atlantic and globally, supporting the natal homing hypothesis in this species as well (Bowen et al. 1993, 1995).

Defining population structure within the western North Atlantic nesting aggregate has been an important management priority since the inception of mtDNA genetic analyses. Bowen et al. (1993) used restriction fragment length ploymorphism (RFLP) analysis on a limited number of samples to tentatively resolve two subpopulations in the southeastern USA: one comprised of rookeries from Florida and another comprised of rookeries north of Florida. Encalada et al. (1998) revisited the question of population structure with additional samples using sequence polymorphisms in a hypervariable segment of the mitochondrial control region. Significant haplotype frequency differences resolved three distinct genetic units within the southeast USA: northwest Florida, south Florida, and northeast Florida-North Carolina. Additionally, samples from Quintana Roo indicated that the Mexican population is genetically distinct from those in the southeastern USA. The low mtDNA diversity in rookeries from northeast Florida to North Carolina provided insufficient resolution to detect any population subdivisions in this aggregate, although such partitions likely exist (Encalada et al. 1998). A total of eight haplotypes were described from western North Atlantic rookeries, including three present only in the Mexican samples.

While the Encalada et al. (1998) survey improved geographical sampling coverage over previous studies, gaps in coverage and low samples sizes in Florida left subpopulation boundaries in question. Pearce (2001) again revisited the population structure of loggerheads in the southeastern USA. This study utilized samples from Encalada et al. (1998), as well as newly collected material, to bring the Florida sample size up to 274 individuals. Pearce (2001) upheld the management units designated in Encalada et al. (1998) and added the Dry Tortugas as a fourth management unit.

Analyses of loggerhead juvenile foraging aggregations and fisheries bycatch utilizing mitochondrial DNA sequence variation have provided strong evidence for trans-ocenaic developmental migrations of loggerheads in the Atlantic and Pacific basins. Carr (1986), following

the suggestions of Brongersma (1972), hypothesized that the large aggregations of juvenile loggerheads in the eastern Atlantic might be attributable to western Atlantic nesting beaches. The majority of individuals comprising oceanic juvenile foraging aggregations off Madeira and the Azores have been linked to rookeries in the western Atlantic using haplotype data, supporting the theory that juveniles from western Atlantic natal beaches may undergo basin-wide oceanic migrations prior to recruiting to neritic habitats (Bolten et al. 1998). Maximum likelihood analysis of samples obtained from long-line fisheries in the Mediterranean suggest that approximately half of the oceanic juveniles sampled could be attributed to western Atlantic nesting beaches, whereas analysis of samples obtained from bottom trawl bycatch supported Mediterranean origin of the neritic juveniles (Laurent et al. 1998). Further analysis of eight foraging aggregations in the eastern Atlantic and Mediterranean [utilizing data from Madeira and the Azores in Bolten et al. (1998) and Lampedusa in Casale et al. (2002)] suggested a clinal shift of haplotype frequencies such that western Atlantic rookeries contribute a higher proportion of juveniles to the western Mediterranean foraging aggregations, whereas the eastern Mediterranean aggregations have a higher proportion of juveniles of Mediterranean origin (Carreras et al. 2006). Pitman (1990) documented a number of juvenile loggerheads off the Pacific coast of Mexico and suggested the aggregation might number in the tens of thousands. The majority of sampled juveniles foraging off Baja California and mortalities from the North Pacific drift-net fishery were attributed to nesting beaches in Japan based on shared RFLP haplotypes (Bowen et al. 1995). This genetic evidence has been corroborated by east-west migration of loggerheads from Baja California to Japan (Resendiz et al. 1998, Nichols et al. 2000).

Analyses of several juvenile foraging grounds in the western Atlantic suggest that the bulk of individuals can be attributed to rookeries in the USA and Mexico. Owing to its large size, the south Florida population was considered the largest contributor to nearly all stocks. Norrgard and Graves (1996), utilizing RFLP techniques, demonstrated that approximately 31% of juveniles sampled in Chesapeake Bay were likely of Florida origin. Bayesian mixed stock

analysis suggested that approximately 67% of loggerheads foraging in the Pamlico-Albemarle Estuarine Complex in North Carolina could be attributed to South Florida beaches (Bass et al. 2004). Similarly, approximately half of the individuals sampled in Charleston Harbor entrance channel were assigned to the Florida nesting population using maximum likelihood analysis (Sears et al. 1995). A maximum likelihood analysis of immature loggerheads captured at the St. Lucie Power Plant canal in southeast Florida suggested that approximately 70% of sampled turtles were attributable to the south Florida subpopulation (Witzell et al. 2002). Bayesian analysis suggests that southeast Florida is the largest contributor of juvenile loggerheads in the Indian River Lagoon in Florida (Reece et al. 2006). Bayesian mixed stock analysis and correlation tests of a large data set representing juvenile feeding aggregations (n=1437) suggest that proximate rookeries do contribute disproportionately to nearby foraging grounds (Bowen et al. 2004).

Loggerheads originating on western North Atlantic beaches face several threats. Terrestrial threats are primarily related to degradation or loss of nesting habitat via development, erosion, beach armoring, or improper beach renourishment (Mosier and Witherington 2002, Steinitz et al. 2001), particularly in Florida. Artificial lighting leads to disorientation events, particularly in hatchlings (Witherington and Bjorndal 1991). In the water, loggerheads face threats from ingestion of marine debris (Tomas et al. 2002) and most notably, incidental capture in various fishing gear, such as coastal gillnet, pelagic longline, and trawl fisheries (Lewison and Crowder 2007).

With all the threats loggerheads face, recovery depends on management to mitigate threats unique to each life history stage. In many cases, subpopulations may be disproportionately affected according to the type and location of the threat. Resolving the number and boundaries of management units within the southeastern USA nesting aggregation is a critical basic step. MtDNA and nuclear DNA analyses are useful tools in characterizing the population structure of these rookeries and providing baseline data for mixed stock analyses. This research builds on a strong legacy and seeks to further earlier work by increasing

samples sizes and beach coverage as well as utilizing additional markers to increase resolving power to make more robust inferences about the integrity of potential management units. In chapter two, I describe the development of a panel of novel, loggerhead-specific microsatellite primers. In chapter three, I address 1) subpopulation designations and management unit affiliations based on mtDNA sequence data and 2) nuclear gene flow among rookeries utilizing the new microsatellite panel.

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CHAPTER 2

TETRANUCLEOTIDE MICROSATELLITES FROM THE LOGGERHEAD SEA TURTLE (*Caretta caretta*)[1]

We describe primers and polymerase chain reaction (PCR) conditions to amplify 15 tetranu-cleotide microsatellite loci from the loggerhead sea turtle (*Caretta caretta*). The primers were tested on 30 individuals that nested along the Georgia (USA) coast. The primer pairs developed in this study yielded an average of 13.9 alleles per locus (range of 10 to 21), an average observed heterozygosity of 0.91 (range 0.79 to 1.00), and an average polymorphic information content of 0.88 (range 0.84 to 0.92).

The threatened loggerhead sea turtle (*Caretta caretta*) occurs globally in warm temperate and tropical ocean basins. Although extensive mitochondrial analyses aimed at characterizing phylogeography and the resolution of management units within this species have been con-ducted (Bowen et al. 1993, 1994, Encalada et al. 1998), complementary microsatellite anal-yses have received comparatively less attention until recently (Bowen et al. 2005). Despite demonstrated conservation of several microsatellite loci across all genera of extant cheloniid turtles (FitzSimmons et al. 1995), there remains a need for additional, highly polymorphic loci to address questions of population genetics. We describe the development of a panel of species-specific, tetranucleotide microsatellite markers that will facilitate, in conjunction with previously developed dinucleotide microsatellite markers, the study of the relationship between kinship and spatial distribution in the loggerhead sea turtle.

We extracted DNA from blood obtained from two nesting (female) loggerheads using the DNAzol reagent (Invitrogen) followed by a phenol-chloroform extraction to remove remaining impurities. We digested DNA with *Rsa* I and *Bst*U I (New England Biolabs) and double-enriched for $(AAAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(AATC)_6$, $(ACAG)_6$, $(ACTG)_6$, $(AAAC)_6$, $(AATG)_6$, $(AGAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(AAAT)_8$, and $(ACAT)_8$ (Glenn and Schable 2005). We ligated enriched product for tetranucleotide repeats into PCR 2.1-TOPO vector, which was used to transform OneShot Top 10 Chemically Competent *Escherichia coli* cells (TOPO TA cloning kit, Invitrogen). We screened 672 colonies for inserts using the β-galactosidase gene and sequenced 672 positive (white) colony PCR products of 500-1100 base pairs using BigDye (PE Applied Biosystems) chemistry and an ABI 3730 sequencer.

Sequences were assembled and edited in Sequencher 4.2 (Gene Codes Corp.) and exported to Ephemeris 1.0 to search for microsatellite repeats. We designed 90 primer pairs for 200 contigs containing unique microsatellite repeats and added an M13-reverse or CAG tag to the 5 end of one of each primer pair using Oligo 4.0 (Molecular Biology Insights) to facilitate fluorescent size detection using ABI sequencers (Boutin-Ganache et al. 2001, Schable et al. 2002). Either a CAG or an M13-reverse tag was chosen for either the forward or reverse primer on the basis of minimizing self-complementarity, pair-complementarity, and secondary structure of each primer or primer-pair. We added GTTT "pigtails" to the 5' end of primers lacking either CAG or M13-reverse tag to facilitate the non-templated addition of adenosine by *Taq* polymerase (Brownstein et al. 1996). We selected 48 primer pairs for testing based on microsatellite repeat number and primer characteristics used in choosing tags.

We optimized primer pairs using DNA samples obtained from loggerheads nesting on several Georgia barrier islands. DNA was extracted from samples using the DNeasy Kit (Qiagen). PCR amplifications were performed in 10 μl volumes using GeneAmp®PCR System 9700 thermal cyclers (PE Applied Biosystems). Final concentrations for optimizing reactions were 10 mM Tris pH 8.4, 50 mM KCl, 0.5 μM pigtailed primer, 0.05 μM CAG or M13-reverse tagged primer (CAG or M13-reverse + primer), 0.45 μM dye labeled tag (HEX or FAM + CAG or M13-reverse), 1.5 mM MgCl2, 0.5 mM dNTPs, 0.5 U Taq DNA Polymerase, and 50 ng DNA. M13 and CAG universal primers were labeled with FAM or HEX fluorescent dyes. Reactions were optimized with 6 individuals using two touchdown thermal cycling programs (Don et al. 1991), each encompassing a 10.5 °C span of annealing temperatures (ranges: 60-49.5°C, 65.0-54.5°C). Cycling parameters were: 21 cycles of 95°C for 20 s; highest annealing temperature for 20 s minus 0.5°C per annealing cycle; and 72°C for 30 s followed by 14 cycles of 95°C for 20 s; 49.5 or 54.5°C, respectively, for 30 s; 72°C for 30 s; and a final extension period of 10 min. at 72°C.

PCR products were checked for amplification and scored using an ABI 3730 sequencer with Genescan Rox500 fluorescent size standard (PE Applied Biosystems). Results were analyzed using GeneMapper software (PE Applied Biosystems) and optimal touchdown cycling schemes were identified. Following optimization, 24 additional individuals were genotyped. We calculated observed and expected heterozygosity and polymorphic information content for each locus using Cervus 2.0 (Marshall et al. 1998). Genepop 3.4 (Raymond and Rousset 1995) was used to test for Hardy-Weinberg equilibrium and genotypic linkage disequilibrium. We conducted a posteriori sequential Bonferroni correction (Rice 1989).

Table 2.1 summarizes the characteristics of 15 primer pairs developed from the loggerhead sea turtle. The number of alleles per locus ranges from 10 to 21, averaging 13.9. Total exclusionary power with both parents unknown is 1.000000. No deviations from Hardy-Weinberg equilibrium or significant linkage disequilibrium were detected after sequential Bonferroni correction.

Acknowledgements

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Table 2.1: Characterization of 15 primer pairs amplifying microsatellite loci from loggerhead sea turtles (*Caretta caretta*) collected along the Georgia (USA) coast. Sequences used to introduce sites for the universal primer are in bold italics. Underlined bases indicate sharing of nucleotides between CAG (5'- CAGTCGGGCGTCATCA-3') tag, M13R (5'- GGAAACAGCTATGACCAT -3') tag, or GTTT 'pigtail' and the locus specific primer binding site. Clone size refers to the predicted size of the PCR product amplified from the clone used to develop each locus; N, number of individuals genotyped at each locus; A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HW}, probability that genotype proportions conform to Hardy-Weinberg equilibrium; and PIC, polymorphic information content.

Locus	Primer Sequence (5'-3')	GenBank Accession Number	Dye	Anneal-ing Start temp. (°C)	Repeats in cloned allele	Clone size (bp)	N	A	Size range (bp)	H_O	H_E	P_{HW}	PIC
Cc1B03 U	**CAGTCGGGCGTC**ATCATCAATAGCTCAGTCATAGGT	DQ917761	FAM	60	ATCT$_{15}$	304	30	13	264-314	0.867	0.868	0.30	0.839
Cc1B03 L	GTTTAGCAGACATAGGGGAGTG												
Cc1F01 U	**CAGTCGGGCGTC**ATCAGTGTGAAGGCTCTAAAACTAAT	DQ917762	FAM	60	ATCT$_{15}$	332	30	13	308-360	0.933	0.904	0.73	0.879
Cc1F01 L	GTTT_ATACTGGGACGATAGGATAAA												
Cc1G02 U	**GGAAACAGCTATGACCAT**AGGTGCCTAAACATTGATAGT	DQ917763	FAM	60	ATCT$_{8}$	286	30	14	262-322	0.900	0.938	0.15	0.917
Cc1G02 L	GTTTATACTGTGCTCTTTCGTGTAAT												
Cc1G03 U	**CAGTCGGGCGTC**ATCAGGAATGTGCAGAATGTATGT	DQ917764	FAM	60	AGAT$_{20}$	324	30	12	276-328	0.933	0.911	0.91	0.886
Cc1G03 L	GTTTAACGAGCATGTATCTAAAGTAA												
Cc1H11 U	**GGAAACAGCTATGACCAT**AACTTTGACTATCTCCCCTAC	DQ917765	HEX	60	ATCT$_{16}$	249	29	12	215-263	0.931	0.887	0.33	0.860
Cc1H11 L	GTTTGACGTGATGCCTGGTGAG												
Cc2G10 U	**CAGTCGGGCGTC**ATCAGTGGCAAGGTCAAATACAG	DQ917766	HEX	60	CTTT$_{18}$	301	30	21	261-326	0.900	0.940	0.89	0.919
Cc2G10 L	GTTT_GCCCTTATTGGTCACAC												
Cc2H12 U	**CAGTCGGGCGTC**ATCATCTTCAGGAGTTTTTGACTTG	DQ917767	FAM	60	AAAG$_9$...AGGC$_9$	325	30	13	294-357	0.967	0.880	0.90	0.852
Cc2H12 L	GTTTCCACACCCCTGTTTCAGA												
Cc5C08 U	GTTTCTTTGATGGTTTTTCTGTTATC	DQ917768	FAM	60	CTTT$_{18}$	325	30	13	281-333	0.933	0.925	0.89	0.903
Cc5C08 L	**CAGTCGGGCGTC**ATCAGTCTTCAGGGTATCGTGTAAT												
Cc5F01 U	GTTTAAAGGATTTGAGATGTTGTATG	DQ917769	HEX	60	CTTT$_{13}$	161	30	21	137-191	0.933	0.930	0.20	0.910
Cc5F01 L	**CAGTCGGGCGTC**ATCACCAGTTGTCTTTCTCCAGTG												
Cc5H07 U	**CAGTCGGGCGTC**ATCA_AGGAAGGAGAGAGGCTTATTAC	DQ917770	FAM	60	CTTT$_{16}$	239	29	11	215-263	1.000	0.889	0.20	0.861
Cc5H07 L	GTTTGGGCAATGAGACTGGAACTA												
Cc7B07 U	GTTTATAATGTTGGTGAGCAATATAG	DQ917771	HEX	65	ATCT$_{18}$	266	27	16	223-299	0.963	0.917	0.65	0.892
Cc7B07 L	**CAGTCGGGCGTC**ATCAGTAAACCAGGCACAGT												
Cc7C04 U	GTTTCCTAACCAACGGAGAAACA	DQ917772	HEX	60	AGAT$_{11}$	226	26	14	202-250	0.962	0.895	0.94	0.866
Cc7C04 L	**CAGTCGGGCGTC**ATCACTCCTTCAGAAGTCTTCACAT												
Cc7E11 U	GTTTGAAGAGCTGACCCCATATAG	DQ917773	FAM	60	AGAT$_{11}$	320	29	11	304-360	0.793	0.869	0.05	0.838
Cc7E11 L	**GGAAACAGCTATGACCAT**AAACACAGAAATGAGGGATAG												
Cc7G11 U	GTTT_CTCAGCATGAAAGTGTAATAC	DQ917774	FAM	60	AGAT$_{14}$	310	30	11	282-322	0.833	0.889	0.61	0.861
Cc7G11 L	**CAGTCGGGCGTC**ATCATGGATTTGTTTCAGTAATAG												
Cc8E07 U	GTTTAGCACTGGGTTGTGTGATTA	DQ917775	FAM	60	CTTT$_{12}$	285	30	12	273-321	0.800	0.888	0.11	0.861
Cc8E07 L	**GGAAACAGCTATGACCAT**ACCACCATTCTGATTTGTTAG												

CHAPTER 3

POPULATION STRUCTURE OF LOGGERHEAD TURTLES (*Caretta caretta*) NESTING IN THE

SOUTHEASTERN USA INFERRED FROM MITOCHONDRIAL DNA SEQUENCES AND

MICROSATELLITE LOCI[1]

ABSTRACT

The loggerhead sea turtle (*Caretta caretta*) is a globally threatened species. The southeastern United States of America (USA) nesting assemblage is one of two primary loggerhead rookeries in the world and the largest in the Atlantic. Determining the stock structure within this nesting aggregation is important for defining management units and providing baseline data for mixed stock analyses of foraging aggregations and stranded turtles. We conducted molecular analyses utilizing mitochondrial DNA (mtDNA) sequence and newly developed microsatellite primers on samples collected from nine rookeries from North Carolina to the Florida panhandle to characterize the population structure of rookeries comprising the southeastern USA nesting assemblage. Pairwise comparisons of mtDNA haplotype frequencies among eight sample sites utilizing genetic distance-based and frequency-based models suggested significant structure among most sites tested in Florida. Pairwise F-statistics and R-statistics revealed no significant nuclear allele frequency differences among any of the nine sample sites. This pattern of complex population structure may be attributable to migration-mediated gene flow among rookeries that are discrete by virtue of female nest site fidelity. However, pairwise tests of genotypic frequency divergence did suggest shallow but significant differences between Georgia and Florida sites and South Carolina and Florida sites, consistent with some level of segregation of northern subpopulation and Florida loggerheads on foraging grounds as inferred from carapace epibiont and tagging studies. Analysis of molecular variance (AMOVA) and pairwise comparisons indicated that the currently designated south Florida management unit may be comprised of multiple management units. More extensive geographical sampling is required to make more robust inferences about the integrity and boundaries of management units.

INTRODUCTION

The threatened loggerhead sea turtle (*Caretta caretta*) occurs globally in warm temperate and tropical ocean basins, although nesting effort is primarily focused on temperate beaches (Bolten 2003). Two primary nesting aggregations of global importance have been identified: the island of Masirah in Oman and the southeastern USA, each hosting more than 10,000 nesting females annually (TEWG 1998, Ross 1998). Within the southeastern USA aggregate, Florida accounts for roughly 90% of total nesting effort (Murphy and Hopkins 1984, Meylan et al. 1995).

Whereas it has long been recognized that a species' genetic diversity and evolutionary potential are often partitioned across its range, enumerating objective criteria for defining distinct conservation units for management purposes has proven difficult. Ryder (1986) introduced the concept of an evolutionary significant unit (ESU), but provided no concrete criteria for defining such units. Moritz (1994) defined recognition criteria for ESUs as reciprocal monophyly for mtDNA and significant divergence at nuclear loci. Moritz (1994) also introduced the concept of management units, which represent a hierarchical level below ESUs, and defined the recognition criteria of management units (MU) as significant divergence of allele frequencies at nuclear or mtDNA loci.

Surveys of mitochondrial DNA (mtDNA) sequence polymorphism globally and in the North Atlantic have provided strong evidence for natal philopatry of nesting females (Bowen et al. 1993, 1994). Loggerheads sampled on nesting beaches within the western North Atlantic all appear to belong to the same ESU, but multiple management units have been described. Sampling in the southeastern USA delineated at least three management units in the southeastern USA nesting aggregate based on mtDNA haplotype frequency divergence: 1) the rookeries from northeast Florida to North Carolina, 2) south Florida, and 3) northwest Florida (Encalada et al. 1998). Pearce (2001) revisited the question of defining management unit boundaries by sequencing additional samples as well as surveying nuclear population structure using five microsatellite loci. Pearce added the Dry Tortugas as an additional

management unit and found an order of magnitude less nuclear structure than mitochondrial structure. There was no significant nuclear divergence among southeastern USA regions using R_{ST} values.

The recovery of the western North Atlantic loggerhead population faces numerous stumbling blocks. Coastal development, beach armoring, artificial lighting, and improper beach renourishment all contribute to degraded or unusable nesting habitat (Witherington and Bjorndal 1991, Steinitz et al. 2001, Mosier and Witherington 2002). Incidental capture in various fishing gear also contributes to substantial losses (Lewison and Crowder 2007). Refining management unit designations and their boundaries within the southeastern USA nesting aggregation is a critical first step in understanding the dynamics of the nesting population and putting fishery-related mortalities into context. The goal of this research is to define the fine scale mitochondrial and nuclear population structure within the southeastern USA nesting aggregation through the application of additional genetic markers and increased sample sizes.

MATERIALS AND METHODS

SAMPLE COLLECTION

Sample sites were chosen to represent all previously described management regions supporting moderate to significant nesting densities in the southeastern USA where beach access and logistics would allow. Sampling locations are listed below with their respective three-digit sample identification codes. Bald Head Island, Brunswick County, North Carolina (BHI); Cape Island (Cape Romain National Wildlife Refuge), Charleston County, South Carolina (CAP); Georgia barrier island samples (GRG) were obtained from: Wassaw National Wildlife Refuge, Ossabaw Island, St. Catherines Island, Blackbeard Island National Wildlife Refuge, Sapelo Island, Jekyll Island, Little Cumberland Island, and Cumberland Island National Seashore; Canaveral National Seashore, Volusia County, northeast Florida

(CAN); Melbourne Beach (Archie Carr National Wildlife Refuge), Brevard County, central Atlantic Florida (CAN); Juno Beach, Palm Beach County, southeast Florida (JUN); Keewaydin Island, Collier County, southwest Florida (KEY); Casey Key, Sarasota County, southwest Florida (MCK); and Cape San Blas, Gulf County, Florida panhandle (CSB). Sampling locations and sample sizes are shown in Figure 3.1 for North and South Carolina, Figure 3.2 for the Georgia barrier islands, and Figure 3.3 for Florida.

Where possible, beaches with on-going saturation tagging projects were chosen as sample sites to maximize sample number and representation for a given region. Saturation tagging is conducted over the entire loggerhead nesting season on BHI, Wassaw Island, Blackbeard Island, Little Cumberland Island, and KEY. Mote Marine Laboratory conducts saturation tagging on MCK during the months of June and July. The University of Central Florida Marine Turtle Research Crew obtained samples while marking nests on five km of beach within the Archie Carr National Wildlife Refuge (MEL). CAP, Ossabaw Island, St. Catherines Island, Sapelo Island, Cumberland Island, and JUN were visited for a single sampling period of less than a week to minimize pseudoreplication. However, poor sample numbers obtained during the initial sampling period necessitated a second sampling period at CAN. Additionally, sampling was conducted on Jekyll Island throughout the nesting season. Microsatellite analysis was used to detect duplicate samples, and they were removed from downstream analyses. Most samples were collected during the 2006 nesting season. However, GRG samples represent the 2005-2006 nesting seasons and CSB samples represent the 2003-2005 nesting seasons.

A 6-mm skin biopsy was obtained from the shoulder region of each nesting female using a sterile, disposable biopsy punch according to techniques described in (Dutton 1996). Samples were stored at room temperature in 2 ml cryovials in 95% ethanol prior to extraction.

DNA EXTRACTION AND PCR AMPLIFICATION

Total DNA was extracted using a Qiagen DNeasy tissue extraction kit. Extractions were performed per Qiagen protocol with the following exceptions: approximately 10 mg of tissue was used, elution buffer was heated to 55° C, and DNA was eluted in separate 100 μl and 50 μl volumes. Following elution, template DNA was diluted 1:1 with 10% Chelex 100 resin (BioRad Laboratories) and boiled for 15 minutes to remove PCR inhibitors. Samples were centrifuged briefly following boiling to pull Chelex beads out of solution. DNA was quantified on a NanoDrop®ND-1000 spectrophotometer.

MITOCHONDRIAL DNA AMPLIFICATION AND SEQUENCING

Polymerase chain reaction (PCR) amplifications of a $\sim$ 390-bp portion of the mitochondrial control region were carried out in 10 μl volumes using primers TCR5 and TCR6 described in Norman et al. (1994). Universal M13 sequencing primers were added to the 5' end of each PCR primer to facilitate sequencing. Final concentrations for reactions were 10 mM Tris pH 8.4, 50 mM KCl, 1.0 μM of each primer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 unit of *Taq* DNA Polymerase, and approximately 25-75 ng of genomic DNA. PCR cycling parameters were as follows: 95°C for 3 minutes; 30 cycles of 95°C for 30 seconds, 50° for 30 seconds, 72°C for 30 seconds; and a final extension of 72°C for 10 minutes. Following amplification, PCR products were purified by adding 2 μl of ExoSap-IT®(USB Corporation) to 5 μl of PCR amplicon and heating according to manufacturer's instructions in a thermocycler. Templates were sequenced in both directions using ABI BigDye chemistry (PE Applied Biosystems) and an ABI 3730 sequencer.

MICROSATELLITE AMPLIFICATION

Microsatellite amplifications were conducted using primers Cc1B03, Cc1F01, Cc1G02, Cc1G03, Cc1H11, Cc2G10, Cc2H12, Cc5C08, Cc5F01, Cc5H07, Cc7B07, Cc7C04, Cc7E11,

Cc7G11, and Cc8E07 (Shamblin et al. in press). PCR amplifications were run in 10 μl volumes using GeneAmp®PCR System 9700 thermal cyclers (PE Applied Biosystems). Final concentrations for reactions were 10 mM Tris pH 8.4, 50 mM KCl, 0.5 μM of each primer, 1.5 mM MgCl$_2$, 0.5 mM dNTPs, 0.5 units of *Taq* DNA Polymerase, and approximately 25-75 ng of template DNA. The 5' end of one primer of each pair was labeled with FAM, HEX, or NED fluorescent dye to facilitate size detection. The sequence "GTTT" was added to the 5 end of each unlabeled primer to facilitate the non-templated addition of adenosine by *Taq* polymerase (Brownstein et al. 1996).

PCR amplifications were conducted using touchdown thermal cycling programs (Don et al. 1991), each encompassing a 10.5°C span of annealing temperatures (ranges: 60-49.5°C, 65.0-54.5°C). Cycling parameters were: 21 cycles of 95 °C for 20 s; highest annealing temperature for 20 s minus 0.5°C per annealing cycle; and 72°C for 30 s followed by 14 cycles of 95°C for 20 s; 49.5 or 54.5°C, respectively, for 30 s; 72°C for 30 s; and a final extension period of 10 min. at 72°C. Initial touchdowns for each primer pair are described in Shamblin et al. (in press).

DATA ANALYSIS

Sequences were aligned, edited, and compared to previously described haplotypes using Sequencher 4.2 (Gene Codes Corporation). Sequences were assigned haplotype designations after nomenclature published on the Archie Carr Center for Sea Turtle Research website (http://accstr.ufl.edu/ccmtdna.html). Samples producing novel or ambiguous sequences were subjected to a second round of DNA extraction, PCR amplification, and sequencing to check for accuracy. Pairwise rookery comparisons and hierarchical analysis of molecular variance (AMOVA) were conducted using Arlequin version 3.1 (Excoffier et al. 2005) to describe the partitioning of genetic variation and test various management unit scenarios. Genetic differentiation was tested using both distance-based and haplotype frequency-based methods.

Genetic distance-based Φ-statistics were calculated using the pairwise model. The indels comprising positions 355 to 360 were treated as a single mutation event. Haplotype frequency-based conventional F-statistics were calculated using a model of equal genetic distance (Weir and Cockerham 1984). Where possible, data reported in Pearce (2001) were compared with those in the present study to test for temporal variation. Additionally, the data from the Dry Tortugas reported in Pearce (2001) were included in alternate runs to determine how the turtles from these islands fit into the current data set.

The sampling localities were initially partitioned into eight sample sites for mitochondrial analyses and nine sample sites for microsatellite analyses. CSB samples were excluded from mitochondrial analyses because data from a previous study were not yet published by collaborators. Because of evidence of moderate inter-island exchange of individuals from tagging data (Richardson et al. 1978, Richardson 1982, Williams and Frick 2001) as well as genotype data (present study), all Georgia samples were pooled for analysis.

Microsatellite alleles were assigned using GeneMapper®version 3.7 software (PE Applied Biosystems). Tests for linkage disequilibrium were conducted for all loci across each population and tests for departure from Hardy-Weinberg equilibrium were conducted with allele frequencies within each population using Arlequin 3.1 (Excoffier et al. 2005). P-values were calculated by Markov chain randomization. Pairwise tests of allele frequency differentiation utilzing F-statistics (Weir and Cockerham 1984) and R-statitics (Slatkin 1995) were conducting using Arlequin 3.1(Excoffier et al. 2005). Additionally, pairwise tests of genotype frequency differentiation were performed with default settings using Genepop 3.4 (Raymond and Rousset 1995). Sequential Bonferroni correction (Rice 1989) was applied to tests to adjust alpha levels for multiple comparisons. Following genotyping of all project samples, 87 samples (representing approximately 15% of the total) were randomly chosen and regenotyped blind to check for genotyping errors.

RESULTS

A 380 base pair segment of the control region was examined after removing ambiguous end bases. Thirty positions were polymorphic, consisting of 22 transitions and eight indels (Table 3.1). Position 358 contains both an indel and a transition. The variable positions resolved 9 distinct haplotypes, one of which was previously undescribed. The new haplotype has undergone an A to G transition at position 119. It has been designated CC-A43 (Genbank acccession number EF396287). All other haplotypes have been previously described from nesting beaches in the southeastern USA (Pearce 2001). The nine haplotypes fall into two distinct clusters as described in Encalada et al. (1998). Haplotypes CC-A1 and CC-A2 account for approximately 94% of individuals typed, but these haplotypes were not uniformly distributed throughout the southeast (Table 3.2).

Analysis of the mtDNA haplotype frequency distributions revealed a pattern of significant structure among the majority of rookeries examined (Table 3.3). Twenty-one of 28 pairwise Φ_{ST} comparisons and 20 of 28 F_{ST} comparisons were significant before Bonferroni correction. The previously defined northern subpopulation rookeries sampled (BHI, CAP, GRG) were fixed for the common haplotype CC-A1, and thus there were no significant comparisons among them. However, there was significant divergence of haplotype frequencies across the Florida peninsula (Figure 3.4). CAN and MEL were not significantly different from each other, but both were significantly different from the northern rookeries, KEY, and JUN. MCK was not significantly different from CAN or MEL, but was significantly different from MCK and JUN prior to correction. Similarly, JUN and MCK were significantly different before correction. After sequential Bonferroni correction, the comparisons between CAN/MEL and JUN remained significant, but those between JUN and KEY and between KEY and MCK were no longer significant. Pairwise intrapopulation comparisons (Φ_{ST} and F_{ST}) of haplotype frequency data reported in Pearce 2001 and the present study from Sarasota County (Casey Key), Keewaydin Island, Melbourne Beach, and Volusia County (Canaveral) yielded no significant differences.

Genetic distance-based and conventional frequency-based AMOVAs were conducted with three different rookery groupings to examine management units. The first AMOVA analysis (scenario A) was conducted by partitioning samples into four groups according to the divisions suggested by corrected pairwise F_{ST} and Φ_{ST} values combined with geographical considerations: northern subpopulation consisting of BHI, CAP, and GRG samples; central Atlantic Florida consisting of CAN and MEL; south Florida consisting of JUN and KEY; and central Gulf represented by MCK. Scenario B partitioned samples into five groups: northern subpopulation, central Atlantic Florida (CAN and MEL), JUN, KEY, and MCK. Scenario C reflected the current management scheme: a northern subpopulation consisting of rookeries from northern Florida to Virginia, and all remaining sampled rookeries grouped into a south Florida management unit. Genetic partitioning among sample individuals within sample sites, among sample sites within management units, and among management units are presented in Table 3.4. The genetic variation accounted for among sample sites within defined management units under the currently recognized management unit arrangement (scenario C) was approximately 12%, whereas the proportion of genetic variation accounted for among sample sites within management units under the proposed scenario A was approximately 1%.

The microsatellites utilized in this study yielded an average of 19.4 alleles per locus at a total exclusionary power of 1.000000 with neither parent known. Locus-specific primer and microsatellite array information are presented in Table 3.5. In tests of Hardy-Weinberg equilibrium, two of 135 locus/population combinations deviated from expected values after sequential Bonferroni correction: MEL, Cc5C08; and KEY, Cc7C04. Seven of 1,035 locus-pair combinations exhibited significant linkage disequilibrium after sequential Bonferroni correction: CAP, Cc1H11 and Cc5F01 (P=0.0001); GRG, Cc1B03 and Cc7G11, (P=0.0000); GRG, Cc1B03 and Cc5H07, (P=0.0001); CAN, Cc2G10 and Cc7C04, (P=0.0001); CAN, Cc1B03 and Cc5H07, (P=0.0003); MCK, Cc1G03 and Cc5C08, (P=0.0001); and CSB, Cc1F01 and Cc1B03, (P=0.0004). There were no consistent patterns of disequilibrium. In tests of allele frequency divergence among sites, none of the pairwise R_{ST} values and only two F_{ST} values

were significant prior to Bonferroni correction (Table 3.3). Neither of these were significant after correction. However, eight of 36 pairwise comparisons of genotypic frequency divergence were significant prior to correction for mulitple tests (Table 3.6). All eight significant comparisons were between either Georgia and Florida sites or South Carolina and Florida sites. Only one of these comparisons (GRG vs. KEY) remained significant after sequential Bonferroni correction.

Of 1305 possible single locus reactions attempted for error testing, 71 comparisons could not be made because of failed amplification in either the original or regenotyped reactions. Of the 1234 comparisons that could be made, there were discrepancies between three single locus genotype comparisons, each at a different locus. All cases involved a single bin shift of a single allele. Two of these were attributed to poor size standard quality in the original genotype. The overall error rate across all loci was 0.243%.

DISCUSSION

The pattern of structure inferred from mitochondrial DNA suggests a shift in haplotype frequencies along both coasts of the Florida peninsula, with haplotype CC-A1 being replaced by CC-A2 from north to south. Because samples were obtained from distant beaches separated by greater than 100 km, the question remains as to how the frequencies change from beach to beach over the continuum. Are there relatively discrete subpopulations with narrow bands of overlap, or do all the beaches between divergent sample sites simply operate on a clinal gradient? Given the relatively consistent nesting densities and geography along each coast between central and southern sample sites, as well as the scale of female site fidelity, one might expect a clinal gradient. However, lack of differentiation between CAN and MEL and marked differentiation between these sites and JUN challenge this notion.

Encalada et al. (1998) predicted that the division between the northern subpopulation and south Florida subpopulation occurs somewhere between Jacksonville and Cape

Canaveral. This is concordant with phylogeographic studies of freshwater and estuarine turtles in the region (Lamb and Avise 1992, Walker and Avise 1998). Nesting densities across these beaches are generally low relative to Volusia County to the south and the Georgia barrier islands to the north. While Amelia Island is clearly associated with the Georgia rookeries based on fixed sharing of haplotype CC-A1 (Encalada et al. 1998, Pearce 2001), the beaches between Amelia Island and Volusia County have not been fully described in terms of mitochondrial DNA haplotype frequencies. The only two samples typed from this region (Jacksonville Beach and Guana River area) were haplotype CC-A1 (Encalada et al. 1998). Analysis of samples from Duval, St. John, and Flagler county beaches should clarify where the boundary should be drawn in terms of delineating the northern subpopulation from rookeries to the south. Regardless of precisely where the line is drawn, the North Carolina, South Carolina, and Georgia rookeries are clearly distinct from those in central and southern Florida in terms of mtDNA haplotype frequencies.

Francisco et al. (1999) provisionally and conservatively considered the Volusia County nesting population a distinct management unit based on the presence and absence of rare haplotypes as compared to the south Florida population and significant haplotype frequency divergence from the northern subpopulation and southern Florida sites. Additional analysis (Pearce 2001) made this distinction less clear as what originally appeared to be an endemic Volusia County haplotype was recovered from the Dry Tortugas off the southwest Florida coast. Additionally, Volusia County was not differentiated from Melbourne to the south. While the Volusia County beaches appeared to be intermediate in terms of haplotype frequencies, Pearce (2001) ultimately suggested grouping them into a south Florida management unit encompassing SW Florida and Atlantic coast beaches to the south. The present study supports an affiliation between the Volusia County nesting population and Melbourne Beach given the nearly identical haplotype frequencies between the sites.

While an overall pattern of clinal shift is supported by the haplotype frequencies along the Atlantic coast of Florida presented in Pearce (2001), sample sizes at some sites were

low. For example, Melbourne was well represented with 46 samples, but Hutchinson Island and Port Everglades, the southern Atlantic sample sites, were represented by 9 and 10 samples, respectively. The present study demonstrates marked haplotype frequency differences between Melbourne Beach and Juno Beach, such that they appear to constitute different stocks for management. While it was possible to test for and confirm no significant haplotype frequency differences between present study samples and those previously collected in Volusia County and Melbourne Beach, this was not the case for Juno Beach samples. Additional samples from Juno Beach as well as samples from Indian River, St. Lucie, and Martin county beaches should permit adequate resolution to determine if there is a consistent clinal gradient over this region or whether central and southern Atlantic beaches might be considered distinct management units with a zone of overlap.

A similar pattern of frequency shift occurs between MCK and KEY on the Gulf coast, though the distinction is more subtle. The similarity in haplotype frequencies between MCK and the central Atlantic sample sites is most likely the product of historical lineage sorting following colonization rather than the result of contemporary demographic connectivity (Pearce 2001). Samples are needed from beaches between MCK and KEY to determine if multiple management units in Southwest Florida are warranted and where boundaries might lie. It will also be informative to examine haplotype frequencies from the remaining nesting beaches to the north in Manatee, Hillsborough, and Pinellas counties. Pearce (2001) determined that the Dry Tortugas constituted a distinct management unit on the basis of haplotype differentiation. Additional samples from the Dry Tortugas were not available for the present analysis. Samples from the Marquesas, which lie between the Dry Tortugas and the remaining Florida keys, as well as the other keys should be examined to determine if they are affiliated with the Dry Tortugas or represent transitional haplotype frequencies between the mainland and Dry Tortugas nesting populations.

The tentative findings of virtually no nuclear population structure among southeastern USA rookeries utilizing allele frequency data support similar earlier inferences (Pearce 2001).

Pearce (2001) suggested three possible mechanisms of this inter-rookery gene flow: males are not natally philopatric to breeding grounds adjacent to nesting beaches, the degree of natal philopatry varies among males, and breeding is occurring at foraging sites or along migration routes where individuals from different rookeries overlap.

With insufficient data available to summarily rule out any of these hypotheses, Australian green turtles offer the best comparable data. Tagging data from the southern Great Barrier Reef demonstrate that males show site fidelity to foraging and breeding grounds (Limpus 1993). Additionally, genetic data from both males and females utilizing breeding grounds off nesting beaches in the Gulf of Carpentaria, northern Great Barrier Reef, and southern Great Barrier Reef provide a test of male philopatry to breeding grounds. Analysis of mitochondrial DNA of females from nesting beaches in each of these regions supported significant population structure among the regions (Norman et al. 1994). Further study of these regions utilizing anonymous nuclear and microsatellite loci suggested moderate gene flow between the Gulf of Carpentaria and the two Great Barrier Reef populations and high gene flow between the two Great Barrier Reef populations (FitzSimmons et al. 1997*b*). Mitochondrial DNA haplotype frequencies of males captured on breeding grounds were comparable to those of nesting females within each respective region, consistent with male philopatry to regional breeding grounds and male-mediated gene flow via mating where foraging and breeding grounds overlap or along migration corridors (FitzSimmons et al. 1997*a*). It should be emphasized that this genetic exchange is "male-mediated" only in the sense that sperm are being exchanged between rookeries while eggs are not (FitzSimmons et al. 1997*a*). Perhaps a more appropriate term would be "migration-mediated" gene flow to reflect the fact that inter-rookery gene flow is facilitated by overlap of both sexes at foraging grounds or on migration routes rather than implying lack of natal philopatry in males. Given this scenario, complete lack of male philopatry in western North Atlantic loggerheads is highly unlikely.

The second hypothesis of reduced philopatry in a percentage of males has some precedence in migratory marine organisms; analysis of pairwise F_{ST} values of summering grounds

of harbour porpoise (*Phocoena phocoena*) suggested females show higher site fidelity overall than males (Rosel et al. 1999). The third hypothesis of breeding during overlap on migration routes or foraging grounds is likely the most robust given the data from Australian green turtles. The inherent difficulty in obtaining samples from reproductively active male logger-heads on breeding grounds and the extensive distribution of the two common haplotypes make direct haplotype frequency comparisons of males and females on breeding grounds difficult. An ongoing inwater study is generating information on the reproductive status and movements of loggerhead males in the Canaveral channel and might soon provide sufficient sample sizes to make preliminary comparisons with the nesting females on adjacent beaches (Blanvillain et al. 2007).

The shallow divergence in microsatellite genotype frequencies between rookeries comprising the previously defined northern and South Florida subpopulations is consistent with other research that suggests segregation of northern and South Florida nesting females on foraging grounds. A study of carapace epibiont communities suggested clear segregation with little overlap of these females on foraging grounds. The transition between divergent epibionts occurred in the area of Flagler and Volusia counties (Caine 1986), which is believed to be roughly concordant with the break between the two subpopulations suggested by mtDNA data analyses. Tagging studies suggest that south Florida nesting females tend to forage in the Gulf of Mexico or in the Bahamas, while northern subpopulation females tend to remain along the Atlantic coast of the USA, rarely moving south of Canaveral (Bell and Richardson 1978, Meylan 1982, Meylan et al. 1983). Satellite telemetry of northern subpopulation females confirms this pattern (Griffin and Murphy 2002, Plotkin and Spotila 2002). There are occasional exceptions, however, including a tag recovery in the Gulf of Mexico of a turtle that nested on Little Cumberland Island, Georgia (Bell and Richardson 1978).

The findings of strong mitochondrial structure and relatively weaker nuclear structure are consistent with earlier results demonstrating complex population structure in this species within the southeastern USA nesting assemblage (Pearce 2001, Bowen et al. 2005). This pat-

tern of complex population structure has been described in the green sea turtle (Karl et al. 1992, Roberts et al. 2004), as well as many migratory marine mammal species (Palumbi and Baker 1994, Brown Gladden et al. 1999, Rosel et al. 1999). As emphasized by Bowen et al. (2005), nuclear connectivity between rookeries does not compromise the integrity of nesting beaches classified as independent subpopulations based on mitochondrial DNA polymorphism. Because of demonstrated female philopatry to natal regions, discrete rookeries survive or perish based on the female component of the population (Avise 1995, Encalada et al. 1998). Thus, regardless of the level of nuclear gene flow and connectivity among rookeries, the fate of the rookery ultimately rests with the success or failure of the female lineage.

The preponderance of the two ubiquitous haplotypes across much of Florida and universal lack of mtDNA variation within the northern management unit (utilizing the TCR5-TCR6 control region sequence) reduce the power of analyses aimed at defining fine scale structure and characterizing mixed foraging stocks. The sharing of a fixed haplotype among nearly all females sampled from North Carolina, South Carolina, Georgia, and northern Atlantic Florida is most likely attributed to founder effect as loggerheads colonized northward into beaches that were unsuitable for nesting during the Wisconsin glaciation that ended approximately 10,000 years before present (Bowen et al. 1993, Encalada et al. 1998). The reduced rate of mtDNA evolution in the Testudines relative to other vertebrates (Avise et al. 1992), along with inferred founder effects, handicap the ability to define management units with sequence data, particularly within the northern management unit. Demographic partitions likely exist on a finer scale than the current mtDNA analyses can resolve. Populations may be geographically and demographically distinct without reflecting genetic divergence because demographic changes can outpace the evolutionary rate of the genetic markers.

Based on data from Pearce (2001) and the present study, the southeastern USA nesting aggregation may be divided into at least six management units: 1) northern subpopulation, encompassing northeast FL through Virginia, 2) central Atlantic Florida, encompassing Volusia and Brevard counties, 3) southeast Atlantic Florida, 4) the Dry Tortugas, 5) south-

western FL, and 6) the Florida panhandle. Samples from additional sites within Florida are required to properly refine boundaries. Moreover, lack of genetic divergence does not preclude the existence of distinct demographic management units. Additionally, while relatively similar nesting densities and "error" in female nest site fidelity may confound partitioning of management units over continuous beaches, demographic partitions may indeed exist. The exchange of a few individuals per generation may be sufficient to prevent genetic divergence but is insufficient to rebuild depleted rookeries on an ecological time scale (Waples 1998). MtDNA variation is only one tool that should be considered in making sound conservation policy decisions about stock structure. The most robust inferences about the integrity and boundaries of management units should be drawn from multiple data sets. Additional exploration of the mitochondrial genome for informative variation, continued characterization of male migratory behavior, and defining the scale of female nest site fidelity utilizing multiple methods should all aid in defining management units within the southeastern USA nesting aggregation.

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Table 3.1: Variable positions of a 380 base pair segment of the mitochondrial control region observed in loggerhead turtles nesting in the southeastern USA. Haplotype designations are based on nomenclature used by the Archie Carr Center for Sea Turtle Research. Sequence numbering begins immediately 3' of primer TCR5 (Norman et al. 1997). '-' indicates deletions.

Haplotype	Variable Positions																													
									1	1	1	1	1	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3
	3	3	3	5	5	6	7	9	0	1	6	6	8	1	3	4	4	5	9	1	1	1	1	2	5	5	5	5	5	6
	2	5	7	1	3	3	8	6	4	9	1	2	8	0	0	4	6	9	4	2	4	5	7	7	5	6	7	8	9	0
CC-A1	T	G	T	T	A	G	A	A	A	C	G	G	C	C	G	C	A	-	A	A	T	A	C	-	-	-	-	-	-	-
CC-A2	C	A	C	C	-	G	G	A	G	A	T	A	G	T	T	A	T	G	G	A	G	C	G	T	G	C	A	A	G	T
CC-A3	C	A	C	C	-	G	G	A	G	A	T	A	A	T	T	A	T	G	G	A	G	C	G	T	G	C	A	A	G	T
CC-A7	C	A	C	C	-	G	G	A	G	A	T	A	G	T	T	A	T	G	G	G	G	C	G	T	G	C	A	A	G	T
CC-A9	C	A	C	T	-	G	G	G	G	A	T	A	G	T	T	A	T	G	G	A	G	C	G	T	G	C	A	A	G	T
CC-A10	C	A	C	C	-	G	G	A	G	A	T	A	G	T	T	A	T	G	G	A	G	C	G	T	G	C	A	G	G	T
CC-A14	T	G	T	T	A	A	A	A	A	C	A	G	C	C	A	C	A	-	A	A	T	A	C	-	-	-	-	-	-	-
CC-A20	T	A	C	C	-	G	G	A	G	A	T	A	G	T	T	A	T	G	G	A	G	C	G	T	G	C	A	A	G	T
CC-A43	C	A	C	C	-	G	G	A	G	G	T	A	G	T	T	A	T	G	G	A	G	C	G	T	G	C	A	A	G	T

Table 3.2: Loggerhead turtle mitochondrial DNA control region haplotypes observed on nesting beaches in the southeastern USA. Beach abbreviations are as follows: Bald Head Island, NC (BHI); Cape Island, SC (CAP); Georgia barrier islands (GRG); Canaveral National Seashore, FL (CAN); Melbourne Beach, FL (MEL); Juno Beach, FL (JUN); Keewaydin Island, FL (KEY); and Casey Key, FL (MCK).

Beach	Haplotype									
	CC-A1	CC-A2	CC-A3	CCA-7	CC-A9	CC-A10	CC-A14	CC-A20	CC-A43	Total
BHI	15									15
CAP	52									52
GRG	153									153
CAN	38	17	1	1			1			58
MEL	70	27	3				2			102
JUN	9	29	5	1		1	1	2	1	49
KEY	17	22						1		40
MCK	35	13	5		1	1	2			57
Total	389	108	14	2	1	2	6	3	1	526

Table 3.3: Pairwise comparisons of genetic differentiation between sample sites. Above the horizontal are estimates of variation for microsatellite loci: R_{ST} (Slatkin 1995) above and F_{ST} (Weir and Cockerham 1984) below. Below the horizontal are estimates of variation for mitochondrial DNA sequence: Φ_{ST} above and F_{ST} below. P values for each test are in parentheses. Bald Head Island, North Carolina (BHI); Cape Island, South Carolina (CAP); Georgia barriers islands (GRG); Canaveral National Seashore, FL (CAN); Melbourne Beach, Fl (MEL); Juno Beach, FL (JUN); Keewaydin Island, Fl (KEY); Casey Key, FL (MCK); Cape San Blas, FL (CSB). CSB samples are represented in microsatellite comparisons but not in mtDNA comparisons. '*' indicates tests that remained significant after sequential Bonferroni correction with a table wide $\alpha = 0.05$.

	BHI	CAP	GRG	CAN	MEL	JUN	KEY	MCK	CSB
BHI	-	0.0024 (0.3459)	-0.0026 (0.5781)	-0.0022 (0.5342)	-0.0013 (0.4896)	-0.0071 (0.7654)	0.0069 (0.2165)	0.0059 (0.2285)	0.0096 (0.1697)
	-	0.0006 (0.3609)	0.0000 (0.3820)	-0.0010 (0.5270)	0.0002 (0.3610)	-0.0007 (0.5025)	0.0000 (0.4065)	-0.0011 (0.6276)	0.0027 (0.1542)
CAP	0.0000 (0.9999)	-	0.0000 (0.4492)	-0.0020 (0.6653)	0.0020 (0.2352)	-0.0025 (0.7164)	-0.0086 (0.9877)	-0.0059 (0.9607)	-0.0039 (0.8123)
	0.0000 (0.9999)	-	-0.0003 (0.5565)	0.0009 (0.1699)	0.0026 (0.0044)	0.0015 (0.1027)	0.0056 (0.2829)	0.0040 (0.3517)	0.0000 (0.4384)
GRG	0.0000 (0.9999)	0.0000 (0.9999)	-	0.0031 (0.1135)	-0.0010 (0.6680)	-0.0024 (0.8016)	-0.0007 (0.5243)	-0.0030 (0.8932)	-0.0015 (0.6503)
	0.0000 (0.9999)	0.0000 (0.9999)	-	0.0007 (0.1302)	0.0004 (0.01461)	-0.0005 (0.6364)	-0.0004 (0.5540)	0.0002 (0.3478)	-0.0016 (0.9394)
CAN	0.2035 (0.0151)	0.3028* (<0.0001)	0.4635* (<0.0001)	-	0.0020 (0.2110)	-0.0069 (0.9858)	-0.0081 (0.9788)	-0.0054 (0.9464)	0.0009 (0.3744)
	0.1791 (0.0010)	0.2741* (<0.0001)	0.4299* (<0.0001)	-	0.0000 (0.3910)	-0.0015 (0.8672)	-0.0019 (0.8709)	-0.0024 (0.9830)	-0.0028 (0.9765)
MEL	0.1642 (0.0190)	0.2283* (<0.0001)	0.3370* (<0.0001)	-0.0110 (0.6701)	-	-0.0012 (0.5945)	0.0052 (0.0996)	0.0003 (0.3964)	-0.0002 (0.4590)
	0.1459 (0.0141)	0.2075* (<0.0001)	0.3112* (<0.0001)	-0.0113 (0.8240)	-	-0.0011 (0.8245)	-0.0025 (0.9809)	-0.0029 (0.9996)	-0.0013 (0.8368)
JUN	0.6995* (<0.0001)	0.7902* (<0.0001)	0.8842* (<0.0001)	0.3426* (<0.0001)	0.3797* (<0.0001)	-	-0.0065 (0.9216)	-0.0054 (0.9364)	0.0001 (0.4538)
	0.5095* (<0.0001)	0.6308* (<0.0001)	0.7777* (<0.0001)	0.2151* (<0.0001)	0.2562* (<0.0001)	-	-0.0021 (0.9586)	-0.0020 (0.9586)	0.0000 (0.4277)
KEY	0.4586* (<0.0001)	0.5990* (<0.0001)	0.7620* (<0.0001)	0.0959 (0.0219)	0.1353* (0.0022)	0.0882 (0.0247)	-	0.0006 (0.4023)	-0.0045 (0.7870)
	0.4342* (0.0001)	0.5761* (<0.0001)	0.7446* (<0.0001)	0.0889 (0.0165)	0.1229* (0.0165)	0.0385 (0.0637)	-	-0.0004 (0.5635)	-0.0019 (0.8210)
MCK	0.2223 (0.0056)	0.3256* (<0.0001)	0.4904* (<0.0001)	-0.0158 (0.7242)	-0.0059 (0.4090)	0.3120* (<0.0001)	0.0745 (0.0326)	-	-0.0040 (0.8062)
	0.1584 (0.0082)	0.2501* (<0.0001)	0.4020* (<0.0001)	-0.0060 (0.4982)	-0.0029 (0.4266)	0.2014* (<0.0001)	0.1020 (0.0057)	-	-0.0018 (0.8928)

Table 3.4: AMOVA table illustrating distribution of molecular variance within the southeastern USA loggerhead turtle nesting assemblage under three different management unit grouping scenarios. Variance partitioning is among groups (AG, where groups are management units), among populations within groups (AP/WG, where populations are individual sample sites), and within populations (WP). Scenario A consists of four management units: northern subpopulation, central Atlantic Florida, southern Florida, and northern southwest Florida. Scenario B consists of five management units: northern subpopulation, central Atlantic Florida, southern Atlantic Florida, southern southwest Florida, and northern southwest Florida. Scenario C reflects the current management unit grouping: northern subpopulation and south Florida (encompassing everything on the peninsula from Volusia County south). Φ-statistics were calculated using a distance matrix calculated with the pairwise model. Conventional F-statistics were calculated using haplotype frequencies.

Scenario	Variance Partitioning	Variance	% Total	φ/F-statistics	P value
A	AG	1.7366	39.99	$\varphi_{CT} = 0.3999$	0.0024
	AP/WG	0.0576	1.33	$\varphi_{SC} = 0.0221$	0.7164
	WP	2.5486	58.69	$\varphi_{ST} = 0.4131$	<0.0001
	AG	0.0765	33.40	$F_{CT} = 0.3340$	0.0025
	AP/WG	0.0012	0.46	$F_{SC} = 0.0070$	0.1747
	WP	0.1515	66.13	$F_{ST} = 0.3387$	<0.0001
B	AG	1.8152	41.97	$\varphi_{CT} = 0.4197$	0.0031
	AP/WG	-0.0391	-0.90	$\varphi_{SC} = -0.0156$	0.6690
	WP	2.5486	58.93	$\varphi_{ST} = 0.4107$	<0.0001
	AG	0.079	34.63	$F_{CT} = 0.3463$	0.0035
	AP/WG	0.041	-1.04	$F_{SC} = -0.0159$	0.8169
	WP	2.5486	66.41	$F_{ST} = 0.3359$	<0.0001
C	AG	1.6205	34.29	$\varphi_{CT} = 0.3429$	0.03593
	AP/WG	0.5564	11.78	$\varphi_{SC} = 0.1792$	<0.0001
	WP	2.5486	53.93	$\varphi_{ST} = 0.4607$	<0.0001
	AG	0.0728	29.54	$F_{CT} = 0.2954$	0.01838
	AP/WG	0.0222	9.01	$F_{SC} = 0.1279$	<0.0001
	WP	0.1515	61.45	$F_{ST} = 0.4133$	<0.0001

Table 3.5: Locus-specific data for 572 female loggerhead turtles nesting in the southeastern USA. N, number of individuals genotyped at each locus; A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HW}, probability that genotype proportions conform to Hardy-Weinberg equilibrium; and PIC, polymorphic information content.

Locus	Dye	N	Proportion Individuals Typed	A	Size Range (bp)	H_0	H_E	P_{HW}	PIC
Cc1B03	NED	555	96.9	22	249-301	0.869	0.869	0.96	0.857
Cc1F01	FAM	547	95.5	15	289-345	0.868	0.885	0.10	0.873
Cc1G02	NED	556	97.0	20	242-316	0.905	0.919	0.14	0.913
Cc1G03	NED	559	97.6	19	262-334	0.921	0.911	0.12	0.903
Cc1H11	FAM	565	98.6	19	188-260	0.894	0.891	0.72	0.881
Cc2G10	FAM	563	98.3	23	245-317	0.913	0.924	0.55	0.918
Cc2H12	FAM	561	97.9	16	276-352	0.857	0.878	0.36	0.864
Cc5C08	HEX	547	95.5	17	264-328	0.898	0.912	0.18	0.904
Cc5F01	FAM	556	97.0	40	111-199	0.942	0.944	0.27	0.940
Cc5H07	FAM	555	96.9	14	198-250	0.867	0.883	0.47	0.871
Cc7B07	HEX	548	95.6	23	206-312	0.916	0.914	0.08	0.907
Cc7C04	FAM	557	97.2	16	185-241	0.907	0.902	0.59	0.893
Cc7E11	FAM	569	99.3	16	282-342	0.856	0.857	0.01	0.841
Cc7G11	HEX	541	94.4	12	269-313	0.869	0.874	0.47	0.860
Cc8E07	NED	569	99.3	19	242-318	0.889	0.891	0.40	0.880

Table 3.6: Genotype frequency differentiation between sample sites expressed as P-values from a Fisher's combined probability test. Comparisons that remained significant after sequential Bonferroni correction are indicated by asterisks. Sample site abbreviations are as follows: Bald Head Island, NC (BHI); Cape Island, SC (CAP); Georgia barrier islands (GRG); Canaveral National Seashore, FL (CAN); Melbourne Beach, FL (MEL); Juno Beach, FL (JUN); Keewaydin Island, FL (KEY); Casey Key, FL (MCK); Cape San Blas, FL (CSB).

|	BHI	CAP	GRG	CAN	MEL	JUN	KEY	MCK	CSB
BHI	-	0.6318	0.5068	0.5226	0.3770	0.7093	0.4954	0.5767	0.1358
CAP	0.6318	-	0.7170	0.0304	0.0058	0.1292	0.0174	0.1262	0.0307
GRG	0.5068	0.7170	-	0.0386	0.0109	0.3365	0.0005*	0.0248	0.1966
CAN	0.5226	0.0304	0.0386	-	0.3092	0.8502	0.8358	0.9383	0.7688
MEL	0.3770	0.0058	0.0109	0.3092	-	0.9677	0.6090	0.9218	0.3352
JUN	0.7093	0.1292	0.3365	0.8502	0.9677	-	0.6057	0.9757	0.4526
KEY	0.4954	0.0174	0.0005	0.8358	0.6090	0.6057	-	0.8560	0.6879
MCK	0.5767	0.1262	0.0248	0.9383	0.9218	0.9757	0.8560	-	0.5055
CSB	0.1358	0.0307	0.1966	0.7688	0.3352	0.4526	0.6879	0.5055	-
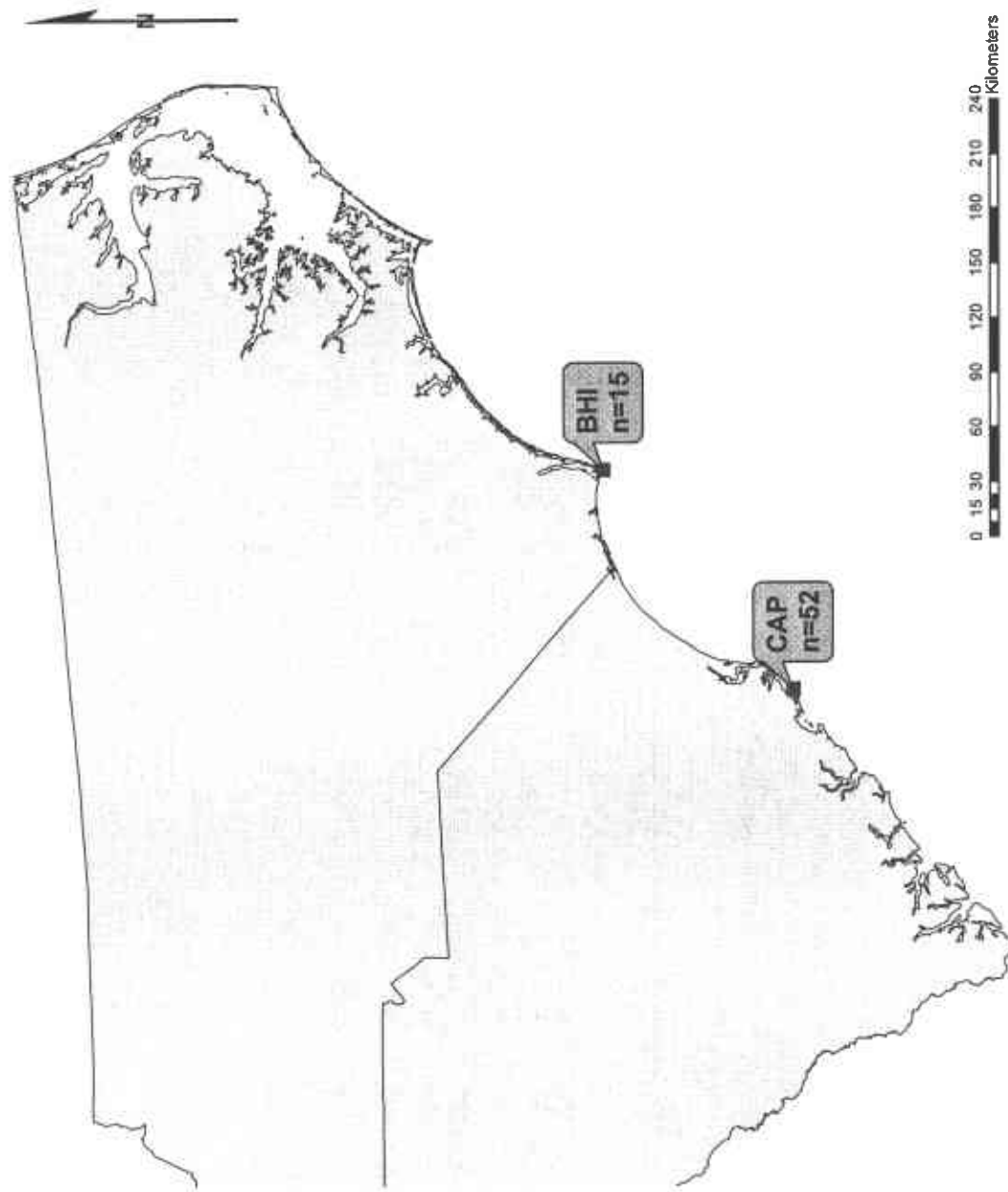
Figure 3.1: Sample beaches and sample sizes of skin biopsies collected during 2006 from nesting loggerhead turtles in the Carolinas. Abbreviations are as follows: Bald Head Island, North Carolina (BHI) and Cape Island, Cape Romain National Wildlife Refuge, South Carolina (CAP).
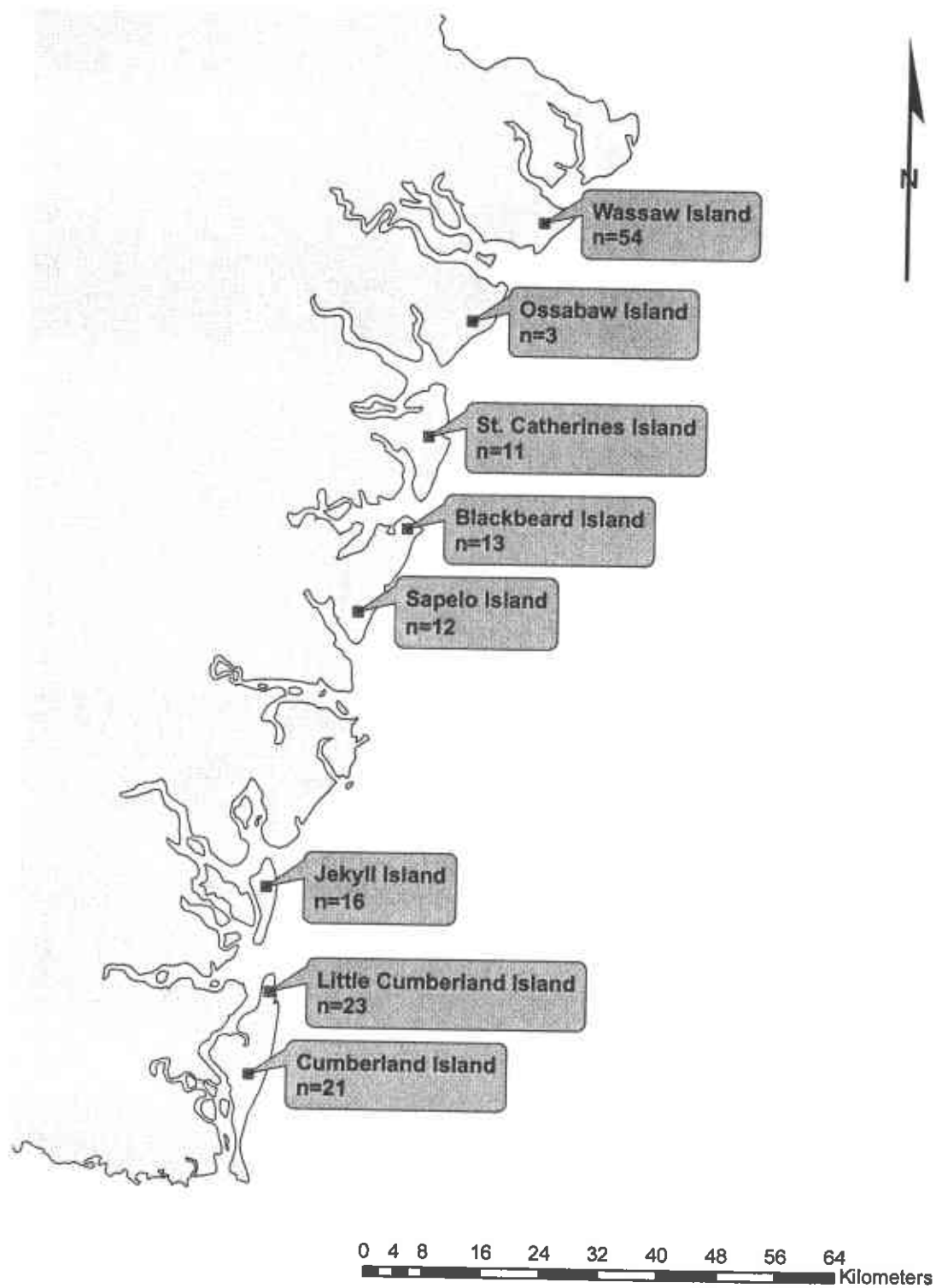
Figure 3.2: Sample beaches and samples sizes of skin biopsies obtained from nesting female loggerhead turtles along the Georgia coast during the 2005 and 2006 nesting seasons.
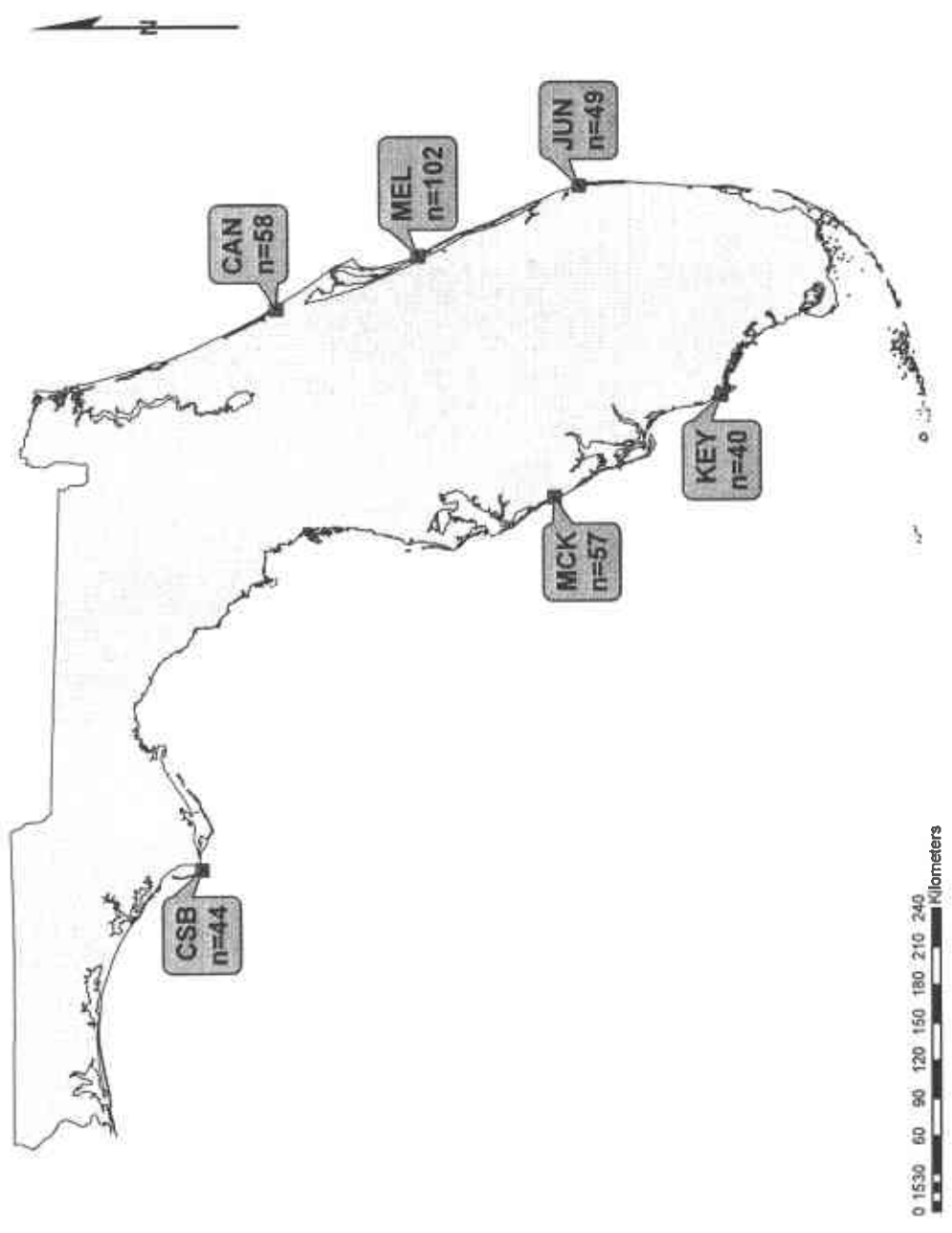
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Figure 3.3: Sample beaches and samples sizes of skin biopsies obtained from nesting loggerhead turtles along the Florida coast. Samples were obtained during the 2006 nesting season except those from CSB, which were collected over the 2003-2005 seasons. Canaveral National Seashore (CAN), Melbourne Beach (MEL), Juno Beach (JUN), Keewaydin Island (KEY), Casey Key (MCK), and Cape San Blas (CSB).
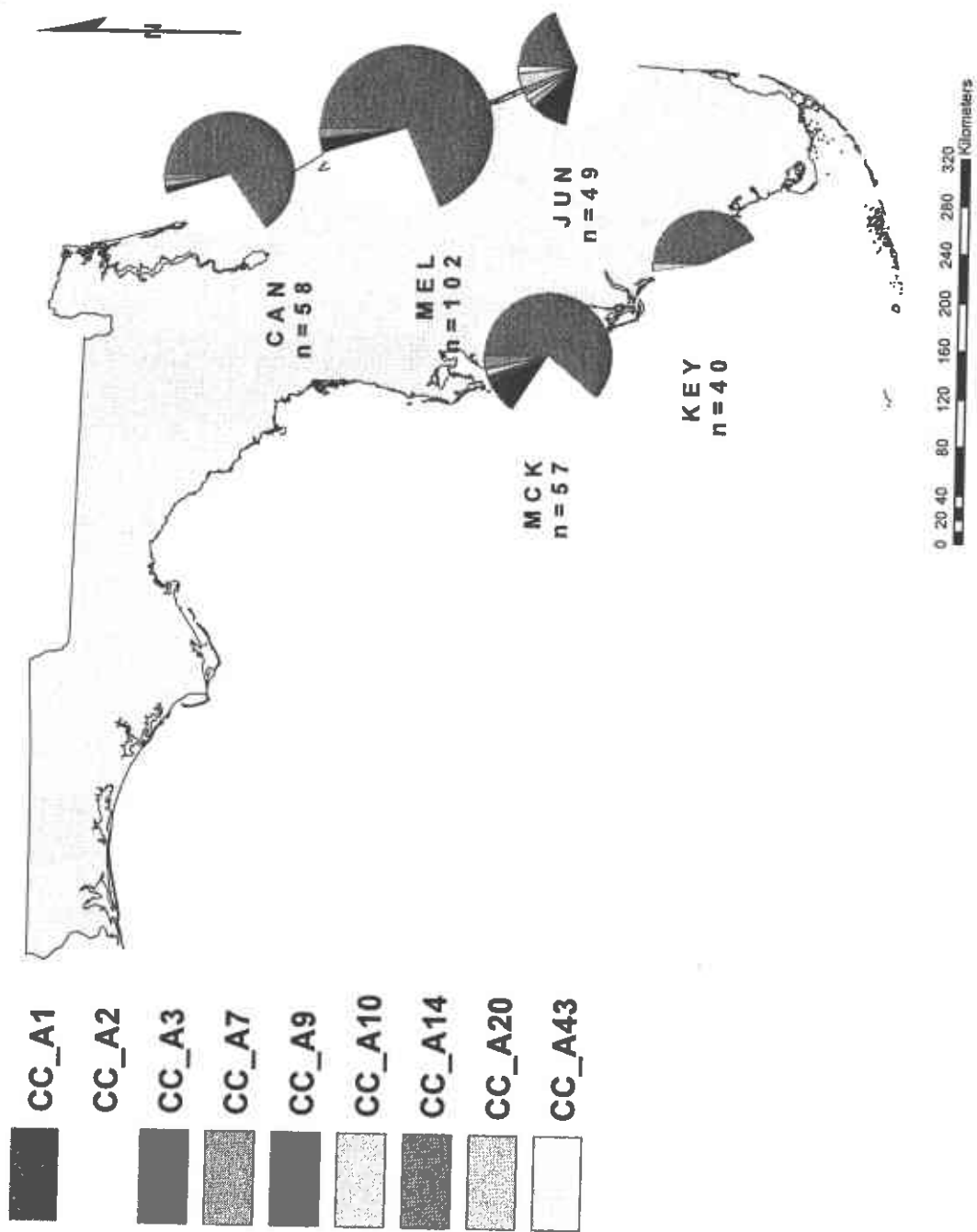
Figure 3.4: Frequencies of loggerhead sea turtle haplotypes utilizing primers TCR5 and TCR6 (Norman et al. 1994) at Florida sample sites. Canaveral National Seashore (CAN), Melbourne Beach (MEL), Juno Beach (JUN), Keewaydin Island (KEY), and Casey Key (MCK). Haplotype designations follow nomenclature from the Archie Carr Center for Sea Turtle Research.

CHAPTER 4

CONCLUSIONS

The microsatellite primers developed for this study are highly polymorphic. They should prove useful in population genetic studies requiring individual resolution, particularly those involving mating systems and relatedness. Some investigators have charged that such highly polymorphic loci may be inappropriate for studies of population structure. O'Reilly et al. (2004) found an inverse relationship between F_{ST} values and microsatellite polymorphism in walleye pollock (*Theragra chalcogramma*) populations across the North Pacific Ocean, presumably due to size homoplasy. Despite such findings, Estoup et al. (2002), who compared size homoplasy within and among populations of honey bees, argue that focus should be on consideration of appropriate population genetic models and use of a reasonably large pool of markers rather than concerns about microsatellite mutation model and size homoplasy. The overall pattern of population structure inferred from microsatellites in the present study is consistent with results from Pearce (2001) with fewer and somewhat less polymorphic markers. F_{ST} and R_{ST} values in the present study did not vary significantly with respect to locus polymorphism. Given these considerations, size homoplasy is likely not a significant confounding factor on the relatively small geographic scale under study. Data from a few additional loci exhibiting less polymorphism might be useful in alleviating any concern over reduced power of the markers to detect structure due to size homoplasy among rookeries or noise that could interfere with the true genetic signal.

The power to detect fine scale subpopulation structure with the mtDNA sequence under study is limited because of low haplotype diversity. In some cases, haplotype frequencies may reflect evolutionary history rather than contemporary gene flow (Avise et al. 2000). Similarly,

inter-rookery female exchange may be sufficient to genetically homogenize populations, but insufficient to replenish depleted stocks (Waples 1998). Limitations of genetic data analysis notwithstanding, care must be taken when making inferences about management units based solely on genetic data (Taylor and Dizon 1999).

Haplotype assignments based on a relatively short segment of mitochondrial control region sequence ($\sim$ 380 bases of $\sim$ 16,500 bases) may have limited utility when assessing population structure on a fine scale given the inferred evolutionary history of the south-eastern United States nesting population. The use of new primers that amplify a larger portion of the control region (Abreu-Grobois et al. 2006) has resulted in additional informative variation in several marine turtle species tested, including loggerheads. Mitochondrial analyses utilizing these primers have succeeded in subdividing common Atlantic and Pacific haplotypes into multiple new haplotypes (Lopez-Jurado et al. 2007, LeRoux et al. 2007). Georgia and Cape Verde loggerheads that were previously undifferentiated using primers TCR5-TCR6 (Norman et al. 1994) show fixed haplotype differences using the new primers (Lopez-Jurado et al. 2007). It is unclear how these additional polymorphic positions will affect the overall picture of population structure in the southeastern United States. Given preliminary results from Cape Verde and the Pacific, searching for additional informative variation in the mitochondrial genome should receive high priority in delineating the fine scale structure of the southeastern United States nesting population as well as examining the mixed stocks utilizing the neritic zone in the western North Atlantic.

Additional inwater studies of adult male loggerheads are required to describe their migratory behavior and reproductive physiology. An ongoing inwater study utilizing satellite telemetry and examining the reproductive status of adult males in the Canaveral channel is generating valuable data about the enigmatic males (Blanvillain et al. 2007). Genetic samples obtained from these individuals and those captured during the 2007 breeding season might provide adequate samples sizes for direct comparisons with nesting females on proxi-

mate nesting beaches. Additional inwater studies at strategically selected sites could provide strong evidence for natal philopatry in male loggerheads.

Nesting beach mark-recapture studies have identified two basic nest site fidelity behaviors: 'alpha' turtles that may nest only once or twice in a season on a given study beach and 'beta' turtles that emerge three or more times (Richardson 1982). Richardson also noted a strong correlation between intraseasonal and interseasonal site fidelity (philopatry) of loggerheads nesting on Little Cumberland Island such that beta turtles were much more likely to be recorded as remigrants. Long distance nesting beach relocations (covering greater than 100 km) have been documented, but appear to represent a small proportion of the population (Bjorndal et al. 1983, LeBuff 1990, Williams and Frick 2001). Properly characterizing the plasticity of these behaviors has important implications for defining the scale of management units as well as generating parameter estimates such as intraseasonal clutch frequency and remigration intervals, which are used to extrapolate population estimates. Alpha turtles may serve as an avenue of gene flow that confounds the discreteness of individual rookeries; however, depending on their proportion of the population and the ultimate scale of site fidelity, the exchange of alpha females among rookeries is likely insufficient to sustain otherwise discrete rookeries. Because of inherent limitations of flipper tagging mark-recapture studies related to the scale of female nest site fidelity and differences in tagging effort among projects, additional research utilizing satellite telemetry and other resources is required to better characterize the scale of female site fidelity and exchange of individual females among beaches.

Results from Pearce (2001) and the present study suggest a minimum of six management units within the southeastern United States nesting aggregate: 1) northern subpopulation, encompassing northeast FL north of Volusia County through Virginia, 2) central Atlantic Florida, encompassing Volusia and Brevard counties, 3) southeast Atlantic Florida, 4) the Dry Tortugas, 5) southwestern FL, and 6) the Florida panhandle. The distinction between Juno and Keewaydin and that between Keewaydin and Casey Key are somewhat ambiguous

given the limitations of the sequence data and data analysis methods employed thus far. These pairwise comparisons are significantly different with Φ-statistics, but are not significant following sequential Bonferroni correction. Despite fixed haplotype sharing between BHI and the other two samples sites within the northern subpopulation (GRG and CAP), pairwise comparisons between BHI and most of the Florida sample sites were not significant after sequential Bonferroni correction. This demonstrates the weakness of the present analysis in detecting differences and suggests that larger sample sizes may be required to make more robust inferences. Additionally, Bonferroni correction may be too stringent and not appropriate for these comparisons.

There may be seven or more management units within the state of Florida (northeast Atlantic, central Atlantic, southern Atlantic, Dry Tortugas, southern southwestern, northern southwestern, and panhandle), but more extensive geographic sampling is required to make robust inferences about the integrity and boundaries of management units. Minimally, the divergence between central and southern Atlantic Florida is compelling. Ongoing analyses and planned sampling during the 2007 nesting season should provide adequate sample coverage to address the genetic component of the management unit question.

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